# From blood to brain

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*DOI:*
10.33612/diss.97724397

# CHAPTER SIX

# The Effect of Tryptophan 2,3-Dioxygenase Inhibition on the Kynurenine Pathway and Cognitive Function in the APP23 Mouse Model of Alzheimer's Disease

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**Manuscript in preparation**

## Abstract

Alzheimer's disease (AD) is associated with progressive endogenous neurotoxicity and hampered inflammatory regulation. The kynurenine (Kyn) pathway, which is controlled by tryptophan 2,3-dioxygenase (TDO), produces neuroactive and anti-inflammatory metabolites. Age-related Kyn pathway activation might contribute to AD pathology and inhibition of TDO was found to reduce AD-related cellular toxicity and behavioural deficits in animal models. To further explore the effect of aging on the Kyn pathway in the context of AD, we analysed Kyn metabolite profiles in serum and brain tissue in the APP23 mouse model of AD. We found that aging had genotype-independent effects on Kyn metabolite profiles in serum, cortex, hippocampus and cerebellum whereas serum concentrations of many Kyn metabolites were reduced in APP23 mice. Next, to further establish the role of TDO in AD-related behavioural deficits, we investigated the effect of long-term pharmacological TDO inhibition on cognitive performance in APP23 mice. Our results indicated that TDO inhibition restored impairments of recognition memory without producing measurable changes in cerebral Kyn pathway activity. TDO inhibition did not affect spatial learning and memory or anxiety-related behaviour. These data indicate that Kyn pathway activation could resemble a cross-species phenotype of aging and warrants further investigation on the role of peripheral Kyn pathway disturbances and cerebral TDO activity in AD pathophysiology.

# Introduction

Late-onset Alzheimer's disease (AD) is the most prevalent cause of dementia and is projected to affect 131 million people worldwide by the year 2050 (Prince et al. 2015). Brains of affected individuals show typical neuropathological features, including gross atrophy of cortical and subcortical brain regions, such as the hippocampus, as well as neurofibrillary tangles consisting of hyperphosphorylated tau protein and amyloid beta (Aβ) plaques. Amongst the mechanisms involved in the pathogenesis of AD are increased activity of N-methyl-D-aspartate (NMDA) receptors, neuroinflammation and disturbances of metabolic homeostasis (Parsons and Raymond 2014; Glass et al. 2010; Jha et al. 2017). The kynurenine (Kyn) pathway, which is activated during aging, has been suggested to play a role in the pathophysiology of AD because it produces metabolites that affect NMDA receptors and is deeply embedded in immunological and metabolic functioning (Lovelace et al. 2017; Maddison and Giorgini 2015).

The Kyn pathway is the primary metabolic pathway of tryptophan (Trp) and is a major source of de novo synthesis of nicotinamide adenine dinucleotide (NAD+) (Liu et al. 2018). The production of Kyn is facilitated by the enzymes tryptophan 2,3-dioygenase (TDO) and indoleamine 2,3-dioxygenase (IDO), which are regulated in a tissue-specific manner by pro-inflammatory cytokines and glucocorticoids (Dostal et al. 2017; Larkin et al. 2016). Metabolites of the Kyn pathway - kynurenines - have diverse biological functions including inflammatory control and metabolic regulation (Cervenka et al. 2017). Certain kynurenines have neuroactive properties mainly because they act on NMDA receptors. The production of these neuroactive kynurenines is cell-type specific (Schwarcz et al. 2012). For example, kynurenic acid (KA), which inhibits the NMDA receptor and is regarded a neuroprotectant, is produced by astrocytes, while quinolinic acid (QA), an NMDA receptor agonist which is generally considered an endogenous neurotoxin, is mainly produced by microglia or infiltrating macrophages (Guillemin et al. 2005; Heyes et al. 1996; Guillemin et al. 2001; Kiss et al. 2003). The production of KA and QA in the brain depends on cerebral TDO/IDO-dependent Trp metabolism and on uptake of Kyn from the blood (Fukui et al. 1991). While aging is associated with increased production/accumulation of the neurotoxin QA in blood and brain (Theofylaktopoulou et al. 2013), AD is additionally associated with reduced levels of the neuroprotectant KA in blood and brain (Giil et al. 2017; Gulaj et al. 2010; Hartai et al. 2007; Widner et al. 2000b). These Kyn

pathway imbalances have been suggested to be involved in AD pathophysiology (Zádori et al. 2018).

The Kyn pathway could be a potential drug target in AD. Augmenting cerebral KA production by inhibition of Kyn metabolism in the blood (thereby increasing Kyn transport to the brain), prevented synaptic loss and improved memory function in a mouse model of AD (Zwilling et al. 2011). In addition, Kyn pathway enzymes in the brain might be targeted directly. TDO is of particular interest in this regard as it is expressed in the hippocampus and is involved in the regulation of adult neurogenesis in mice (Ohira et al. 2010; Kanai et al. 2009). Neurogenesis within the hippocampus plays a crucial role in memory formation and is affected early in the course of AD (Lazarov and Hollands 2016). Hippocampal TDO expression was found to be activated by Aβ oligomers (Woodling et al. 2016) and TDO expression was increased in hippocampal regions of AD patients (Wu et al. 2013). Inhibition of TDO prevented neurodegeneration in Caenorhabditis elegans and Drosophila melanogaster models of AD (van der Goot et al. 2012; Breda et al. 2016). In C. elegans, this protection was independent of Kyn metabolites, while in Drosophila protection was established by increasing relative levels of KA. Additionally, in 3-month-old mice that express mouse/human amyloid precursor protein (APP) - which is cleaved to produce Aβ - and mutant human presenilin 1 (APP/PS1) - which is involved in cleavage of APP -, a four-week treatment with a TDO inhibitor prevented deficits in hippocampal-dependent memory function (Woodling et al. 2016). Taken together, these data suggest that TDO could be an interesting drug target in AD.

In this study, we investigated the effect of aging on Kyn metabolite profiles in blood and brain of the APP23 amyloidosis mouse model. Our analyses reveal diverse disturbances with aging that show overlap with age-related kynurenine changes in humans. Next, we investigated the effect of long-term oral administration of a TDO inhibitor on Kyn metabolite profiles and cognitive functioning in APP23 mice. In our hands, TDO inhibition had minor effects on Kyn metabolites in blood and brain, but improved recognition memory, while not affecting anxiety-related behaviour and spatial learning and memory. These data suggest that age-related alterations of the Kyn pathway could be a cross-species phenotype of aging and warrant further investigation of TDO as a drug target in AD.

# Methods

## Animals

APP23 mice, which express mutated human APP (Swedish double mutation; K670N/M671L) under control of a murine Thy1 promotor, were obtained as previously described (Sturchler-Pierrat et al. 1997). Colonies were maintained on a C57BL/6J background and backcrossed for at least 20 generations using PCR to establish genotypes. For the current study, we used male heterozygous APP23 and wild-type littermate mice housed together in a conventional laboratory environment with a 12 h light/dark cycle, constant room temperature and humidity, and free access to food and water. The study protocol was approved by the animal ethics committee of the University of Antwerp (reference number 2018-33) and was in compliance with the European Communities Council Directive on the protection of animals used for scientific purposes (2010/63/EU).

## Materials

Dimethyl sulphoxide (DMSO) and the TDO inhibitor 680C91 were purchased from Sigma-Aldrich. Stock solutions of vehicle (VEH) and 680C91 (100mM DMSO) were made and stored at -20 □C for a maximum duration of three weeks. Directly prior to use, stock solutions were diluted in water (pH 3.2) with DMSO at a final concentration of 4.2%.

## Experimental design

For the first part of the study focusing on HPLC/MS-MS analysis of kynurenine metabolites, untreated mice were sacrificed at 3 months (APP23 n = 4; wild-type n = 5), 6 months (APP23 n = 4; wild-type n = 5) and 12 months of age (APP23 n = 5 and wild-type n = 5).

For the second part of the study, 6-moonth-old wild-type and APP23 mice were treated with vehicle (VEH) or VEH + 680C91 (7.5 mg/kg) via oral gavage (7.5 ml/kg) for six days in the week between 9:30 am and 10:30 am for the period of six weeks (wild-type VEH n = 13; wild-type VEH + 680C91 n = 12; APP23 VEH n = 8; APP23 VEH + 680C91 n = 8). Behavioural experiments were performed from week four onward in a predetermined order and on fixed hours starting with the Morris water maze, followed by the novel object recognition (NOR) test. Next, the light/dark cycle was reversed and – after a five-

day adaptation period – the open field and elevated plus maze tests were performed on consecutive days. Mice were sacrificed four hours after the final treatment.

## Behavioural experiments

Mice were allowed to adapt to the experimental room for at least one hour prior to the start of the experiments, which were performed by a single experimenter. All experiments were recorded and analysed using a video-tracking system (Ethovision, Noldus, The Netherlands).

### *Open field*

Exploratory and anxiety-related behaviour in the open field was measured for each mouse individually for 5 min during the dark phase of the animal's activity cycle in a brightly lit arena (50 cm x 50 cm). Mice always started from the same corner and were allowed 1 min of adaptation. Path length and location parameters, including the number of entries and time spent in the 7 cm x 7 cm corners and the centre circle were recorded.

### *Elevated plus maze*

The elevated plus maze was used to further assess anxiety-related behaviour. The setup consisted of a cross-shaped maze with a central area giving access to each arm 30 cm in length and 5 cm in width. Two opposing arms were enclosed by 30 cm high walls while the other opposing arms were not enclosed by walls. The maze was placed 60 cm above the floor. Mice were placed in the central area facing the left enclosed arm. Trajectories were recorded during 5 min and the number of entries into the different arms and the time spent in the different arms were calculated.

### *Morris water maze*

The MWM was performed to assess hippocampus-dependent spatial learning and memory (D'Hooge and De Deyn 2001). The setup consisted of a circular tank (diameter 150 cm, height 30 cm) filled with water that was opacified using non-toxic white paint and kept at 25 □C. Invariable visual cues were placed around the pool. The MWM consisted of an acquisition phase and a probe trial. The acquisition phase was performed over the period of four days and consisted of two daily trial blocks (one at 10:30 and one at 15:00) of four trials with a 15 min inter-trial interval. During the acquisition phase, a round acrylic glass platform (diameter 15 cm) was placed 1 cm below the water surface

on a fixed position in the centre of one of the pool's quadrants. Mice were placed in the water and were recorded while trying to find the hidden platform for a maximum duration of 120 s. The starting positions varied in a semi-random order. Mice that did not succeed to find the hidden platform within the given amount of time were guided to the platform before being returned to their cage. The probe trial followed four days after the final acquisition trial. For this trial the platform was removed, mice were placed in the tank at a fixed position and swimming trajectories were recorded during a period of 100 s. The total path length until the platform was calculated for each trial block during the acquisition phase. The average distance to the target position (prior position of the platform) and the time spent in each quadrant (target, adjacent 1, adjacent 2 and opposite) were calculated for the probe trial.

### *Novel object recognition*

The NOR test was performed to assess recognition memory and was performed during four consecutive days (Ennaceur and Delacour 1988). On the first two days of the protocol, mice were individually habituated to an empty arena (40 cm x 24 cm) during 10 min. On the third day (familiarization phase), two identical objects (brown-coloured flasks) were placed 10 cm apart in the centre of the arena and mice were allowed to freely explore the cage and objects for 5 min. On the fourth day (novel object phase) one object was replaced with a novel object (different colour and shape, but similar in size). Mice were then placed in the arena and again allowed to explore for 5 min. To reduce anxiety, mice were tested during the light phase. Trajectories and nose-point locations were recorded. Exploration time was defined as the time during which the nose-point was directed towards one of the objects with a proximity of 3 cm. The recognition index (time spent exploring novel object divided by total time exploring both objects) was calculated as a measure of recognition (Antunes and Biala 2012).

## HPLC/MS-MS analysis of kynurenine metabolites

Mice were anesthetized with a mixture of ketamine-xylazine (100 mg/kg and 20 mg/kg, respectively in a total volume of 10 ml/kg) administered intraperitoneally. Blood was drawn by retro-orbital puncture with a glass capillary and allowed to coagulate for at least 30 min at room temperature. Serum was separated by centrifuging at 1500 x g for 10 min and subsequently stored at 80 °C. A thoracotomy was performed followed by whole body transcardial perfusion with PBS for 5 min. Brain regions (bilateral hippocampi,

cortex and cerebellum) were dissected on ice and samples were immediately snap frozen in liquid nitrogen and stored at -80 °C until further analysis. Samples were then homogenized in 1 ml ice-cold 0.1M acetic acid using bead homogenization at 4 °C and centrifuged at 12000 g for 15 min. The supernatant was collected and stored at -80 °C. Concentrations of Trp and Kyn pathway metabolites (Kyn, 3-Hk (3-hydroxykynurenine), AA (anthranilic acid), KA, XA (xanthurenic acid), QA, 3-HAA (3-hydroxyanthranilic acid) and PA (picolinic acid)) were measured using isotope dilution mass spectrometry.

### Statistical analysis

Statistical analyses were performed using IBM SPSS statistics 24 (IBM Corp, 2014) and JMP Pro 14. One- or two-way ANOVAs were conducted to investigate the effect of age and genotype or treatment and genotype and their interaction on metabolite concentrations with Tukey post-hoc tests in the case of significant interaction. Planned contrasts were used to investigate whether metabolites followed a linear or quadratic trend with aging. Paired t-tests were used to investigate differences between the two days of novel object test and two-way repeated measures ANOVA in combination with two-way ANOVA was conducted to analyse Morris water maze learning phase data. The criterion α was set to .050 for all tests of significance.

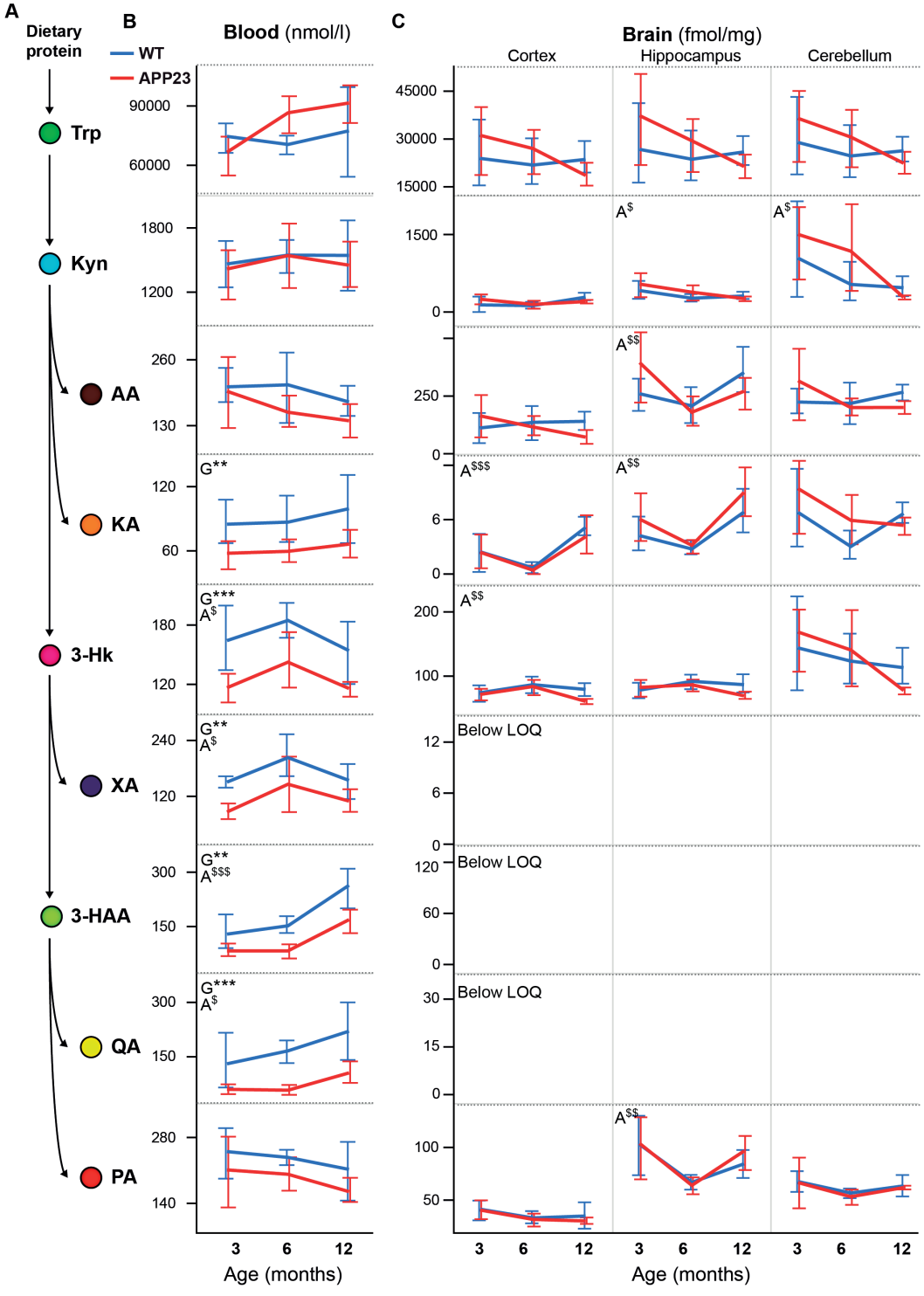
## Results

### Peripheral kynurenine pathway activity is dysregulated in APP23 mice compared to wild-type but is similarly affected by aging

Age-related activation of the Kyn pathway - measured in blood - is common in humans and could contribute to Kyn pathway dysregulation in neurodegeneration. To characterize the effect of aging on Kyn pathway activity in mice in the context of

**► Figure 1. Reduction of kynurenine pathway metabolites in the blood in APP23 mice and genotype-independent kynurenine pathway alterations in blood and brain during aging**

**A.** Simplified representation of Trp metabolism along the Kyn pathway. **B.** Line plots showing median concentrations of metabolites in blood and (**C**) cortex, hippocampus and cerebellum from 3-, 6- and 12-month-old wild-type (blue symbols) and APP23 (red symbols) mice (n=4-5). Error bars represent interquartile ranges. Significance of the main effect of genotype (G) or linear/quadratic effect of aging (A) in ANOVA are depicted using * and $ respectively. */$ p<.05; **/$$ p<.01; ***/$$$ p<.001. **Abbreviations**: Trp, Tryptophan; Kyn, Kynurenine; AA, anthranilic acid; KA, kynurenic acid; 3-Hk, 3-hydroxykynurenine; XA, xanthurenic acid; 3-HAA, 3-hydroxyanthranilic acid; QA, quinolinic acid; PA, picolinic acid.


A
Dietary protein
Trp
Kyn
AA
KA
3-Hk
XA
3-HAA
QA
PA
B
Blood (nmol/l)
WT
APP23
90000
60000
1800
1200
260
130
G**
120
60
G***
A$
180
120
G**
A$
240
120
G**
A$$$
300
150
G***
A$
300
150
280
140
3
6
12
Age (months)
C
Brain (fmol/mg)
Cortex
Hippocampus
Cerebellum
45000
30000
15000
1500
0
A$
A$
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250
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Age (months)

neurodegeneration, we analysed kynurenines in the serum of APP23 and wild-type mice at 3-, 6- and 12-month-old (**Figure 1B**).

Our analyses showed that age did not interact with genotype to affect serum concentrations of Kyn metabolites. Regarding the effect of genotype, our analyses revealed reduced concentrations of KA ($F(1,28)=10.3$, $p=.004$), 3-Hk ($F(1,28)=17.3$, $p<.001$), XA ($F(1,27)=10.7$, $p=.004$), 3-HAA ($F(1,28)=14.5$, $p=.001$) and QA ($F(1,27)=22.3$, $p<.001$) in serum of APP23 mice compared to wild-type with differences ranging from 25% (KA) to 56% (QA).

Aging was associated with changes in concentrations of XA ($F(2,27)=3.9$, $p=.037$), 3-HAA ($F(2,28)= 15.1$, $p<.001$) and QA ($F(2,27)= 3.8$, $p=.038$) in serum of wild-type and APP23 mice. Planned-contrast testing revealed differential trends between aging and these kynurenines with a quadratic trend peaking at 6 months for XA ($p=.012$), a trend with both a quadratic and linear component with a peak at 12 months for 3-HAA ($p<.001$ and $p=.037$ respectively) and a linear increase of QA ($p=.015$).

These results suggest dysregulated peripheral Kyn pathway activity in APP23 mice, which is characterized by reduced concentrations of several downstream Kyn metabolites. In addition, aging causes changes in peripheral Kyn pathway activity in mice that are similar in APP23 and wild-type.

## Kynurenine pathway activity is not affected in the brain of APP23 mice and shows region-specific changes during aging

Because certain kynurenines can cross the blood-brain barrier, dysregulation of peripheral Kyn pathway activity can result in altered Kyn pathway activity in the brain (Fukui et al. 1991). To analyse cerebral Kyn pathway activity, we analysed Kyn metabolites in the cortex, hippocampus and cerebellum in 3-, 6- and 12-month-old APP23 and wild-type mice (**Figure 1C**).

Similar to our results in serum, interaction analyses indicated no interaction between age and genotype for Kyn metabolite concentrations in cortex, hippocampus or cerebellum. In addition, no differences in the concentrations of kynurenines were noted in any of the investigated brain regions between APP23 and wild-type mice.

Regarding the effect of age, our analyses showed age-related trends of Kyn pathway changes in the cortex, hippocampus and cerebellum. Hippocampal Kyn pathway activity varied most strongly during aging with changes in levels of Kyn ($F(2,28)=4.24$, $p=.028$), AA ($F(2,28)=4.53$, $p=.023$), KA ($F(2,24)=7.37$, $p=.005$) and PA ($F(2, 24)=7.37$, $p=.005$). Planned-contrast testing indicated a linear decrease of Kyn ($p=.011$), quadratic trends with low concentrations at 6 months for AA and PA ($p=.007$ and $p=.001$ respectively) and a trend with both a quadratic and linear component peaking at 12 months for KA ($p=.034$ and $p=.009$ respectively). In the cortex, aging was associated with alterations of KA ($F(2, 27)=12.3$, $p<.001$) and 3-Hk ($F(2,28)=4.3$, $p=.027$) which were both characterized by a quadratic trend ($p=.009$ and $p=.001$ respectively). For the cerebellum aging was associated with changes in Kyn ($F(2,28)=3.79$, $p=.039$) with a linear decrease during aging ($p=.012$).

Taken together, these data provide evidence of Kyn pathway alterations during aging in the brain of APP23 and wild-type mice, which are most evident in the hippocampus. Surprisingly, although several kynurenines were reduced in the serum of APP23 mice, this was not paralleled by changes in cerebral metabolite concentrations.

## Long-term TDO inhibition specifically improves recognition memory in APP23 mice

Studies in C. elegans and Drosophila melanogaster have shown that TDO inhibition can reduce Aβ toxicity (van der Goot et al. 2012; Breda et al. 2016). In addition, it was shown that oral administration of the TDO inhibitor 680C91 improved recognition memory in a mouse model of AD mice (Woodling et al. 2016). To further establish the role of TDO in memory function in the context of AD, we studied the effect of long-term oral administration of 680C91 on learning and memory in 6-month old APP23 and wild-type mice.

To establish whether TDO inhibition could similarly improve recognition memory in APP23 mice, we conducted the novel object recognition test. During the familiarization phase, all groups of mice spent an equal amount of time exploring both objects (data not shown). During the test phase, wild-type mice showed interest in the novel object which was not affected by treatment ($p<.001$ for vehicle and $p=.002$ for 680C91 for paired t-test). However, vehicle-treated APP23 mice showed impaired novel object recognition

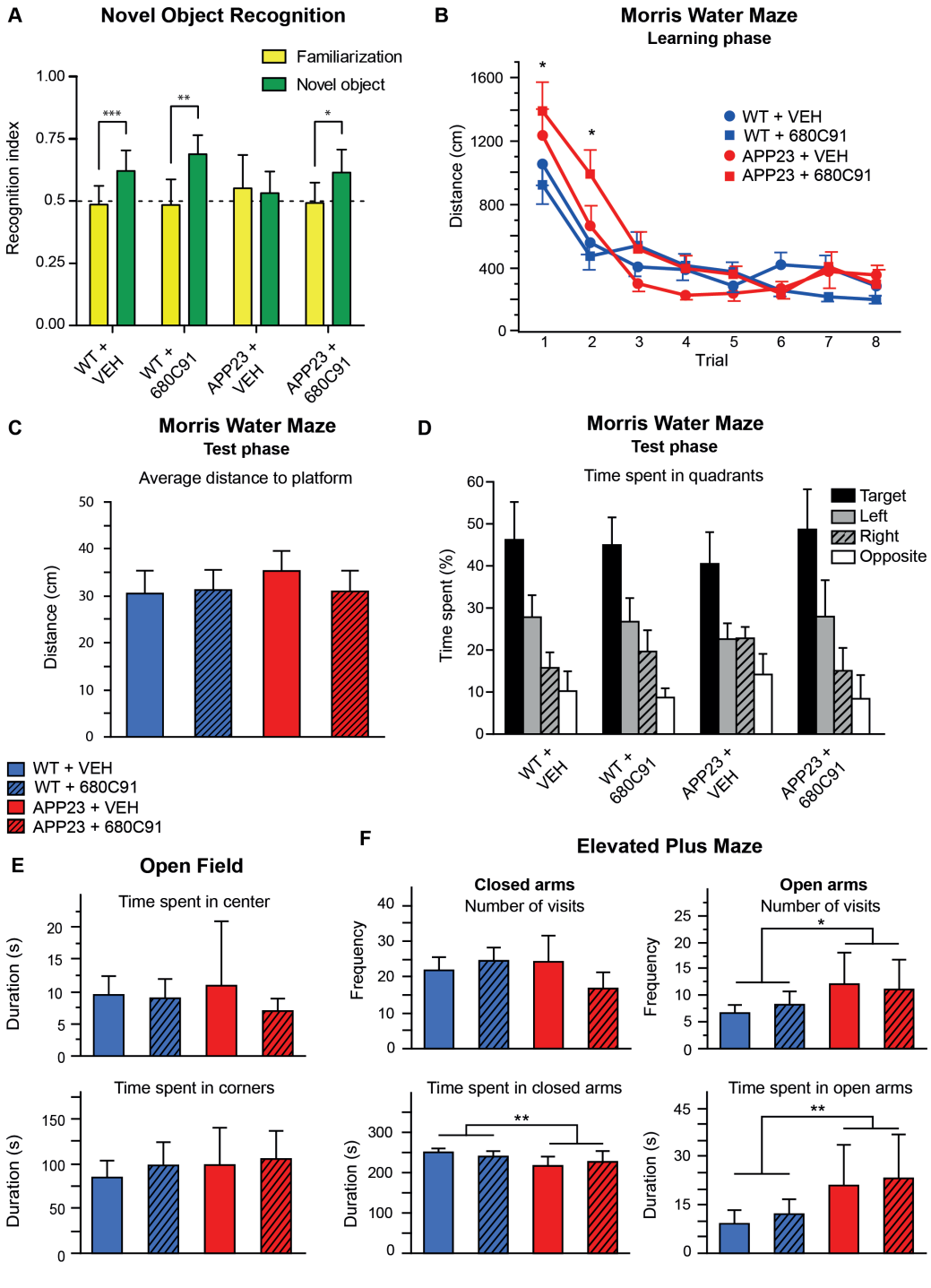
(p=.787 for paired t-test) which could be restored by 680C91 treatment (p=.013) (**Figure 2A**).

Next, to analyse whether TDO inhibition could also improve spatial learning and memory, APP23 and wild-type mice were trained during eight trial blocks in the Morris water maze. Repeated measures ANOVA indicated that mice were able to find the platform more easily with each consecutive trial block ($F(7,259)=54.9$, $p<.001$) (**Figure 2B**). Although APP23 mice spent a longer distance to find the platform during the first two trial blocks, all groups of mice similarly learned the location of the platform during the final trials suggesting no learning impairment in APP23 mice in this setup-up and no effect of 680C91 treatment. A probe trial was conducted four days after the final trial block to assess spatial memory. The average distance to the specific target location during the probe trial was similar between the groups (**Figure 2C**). However, following up on a significant treatment-by-genotype effect ($F(1,40)=8.0$, $p=.007$), post-hoc analyses indicated that APP23 mice treated with 680C91 spent less time in the quadrant on the right side of the target quadrant compared to wild-type mice ($p=.020$), suggesting a subtle difference in the search patterns of these mice (**Figure 2D**).

Taken together, impaired recognition memory in APP23 mice could be rescued by long-term oral administration of the TDO inhibitor 680C91 while spatial learning and memory

**► Figure 2. Inhibition of tryptophan 2,3-dioxygenase improves hippocampal-based recognition memory but does not influence measures of anxiety and spatial learning and memory in APP23 mice**

**A.** Graph showing recognition index during a 5-minute familiarization phase (time spent exploring familiar object 1/time spent exploring familiar objects 1 and 2) versus the recognition index during the novel object phase 24 hours later (time spent exploring novel object/time spent exploring both objects). Results from paired-sample T tests are shown. * p<.05; ** p<.01; *** p<.001. **B.** Graph displaying the mean and standard errors of the total distance travelled during eight consequent trials during the learning phase of the Morris water maze. Results from main effect of genotype by two-way ANOVA are depicted. * p<.05. **C.** Graph showing the average distance to the platform location during the Morris water maze test phase (probe trial). **D.** showing the percentage time spent in the different quadrants the during the Morris water maze test phase (probe trial). Results from Tukey post-hoc test are depicted. * p<.05. **E.** Line bars showing mean and 95% CI of the total time spent in the centre and corners during the open field test paradigm in mice treated with vehicle (DMSO) or TDO inhibitor (680C91) through oral gavage (n=8-13). **F.** Line bars showing mean and 95% CI of the visiting frequency and the duration spent in the closed and open arms of the elevated plus maze. Results from two-way ANOVA are depicted. * p<.05; ** p<.01.


A
Novel Object Recognition
Familiarization
Novel object
Recognition index
1.00
0.75
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0.00
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**
*
WT + VEH
WT + 680C91
APP23 + VEH
APP23 + 680C91
B
Morris Water Maze
Learning phase
Distance (cm)
1600
1200
800
400
0
WT + VEH
WT + 680C91
APP23 + VEH
APP23 + 680C91
1
2
3
4
5
6
7
8
Trial
C
Morris Water Maze
Test phase
Average distance to platform
Distance (cm)
50
40
30
20
10
0
WT + VEH
WT + 680C91
APP23 + VEH
APP23 + 680C91
D
Morris Water Maze
Test phase
Time spent in quadrants
Time spent (%)
60
50
40
30
20
10
0
Target
Left
Right
Opposite
WT + VEH
WT + 680C91
APP23 + VEH
APP23 + 680C91
E
Open Field
Time spent in center
Duration (s)
20
15
10
5
0
Time spent in corners
Duration (s)
150
100
50
0
F
Elevated Plus Maze
Closed arms
Number of visits
Frequency
40
30
20
10
0
Open arms
Number of visits
*
Frequency
25
20
15
10
5
0
Time spent in closed arms
**
Duration (s)
300
200
100
0
Time spent in open arms
**
Duration (s)
45
30
15
0

were not impaired in APP23 mice and were not affected by 680C91 treatment in the current experimental set-up. These data suggest a role for TDO in specific types of memory in APP23 mice.

## Long-term TDO inhibition does not affect anxiety in APP23 mice

Genetic inhibition of TDO was found to reduce anxiety-related behaviour in mice (Kanai et al. 2009; Too et al. 2016). As anxiety could influence the performance of APP23 mice during memory test, we next investigated the effect of long-term TDO inhibition on anxiety-related behaviour in APP23 and wild-type mice.

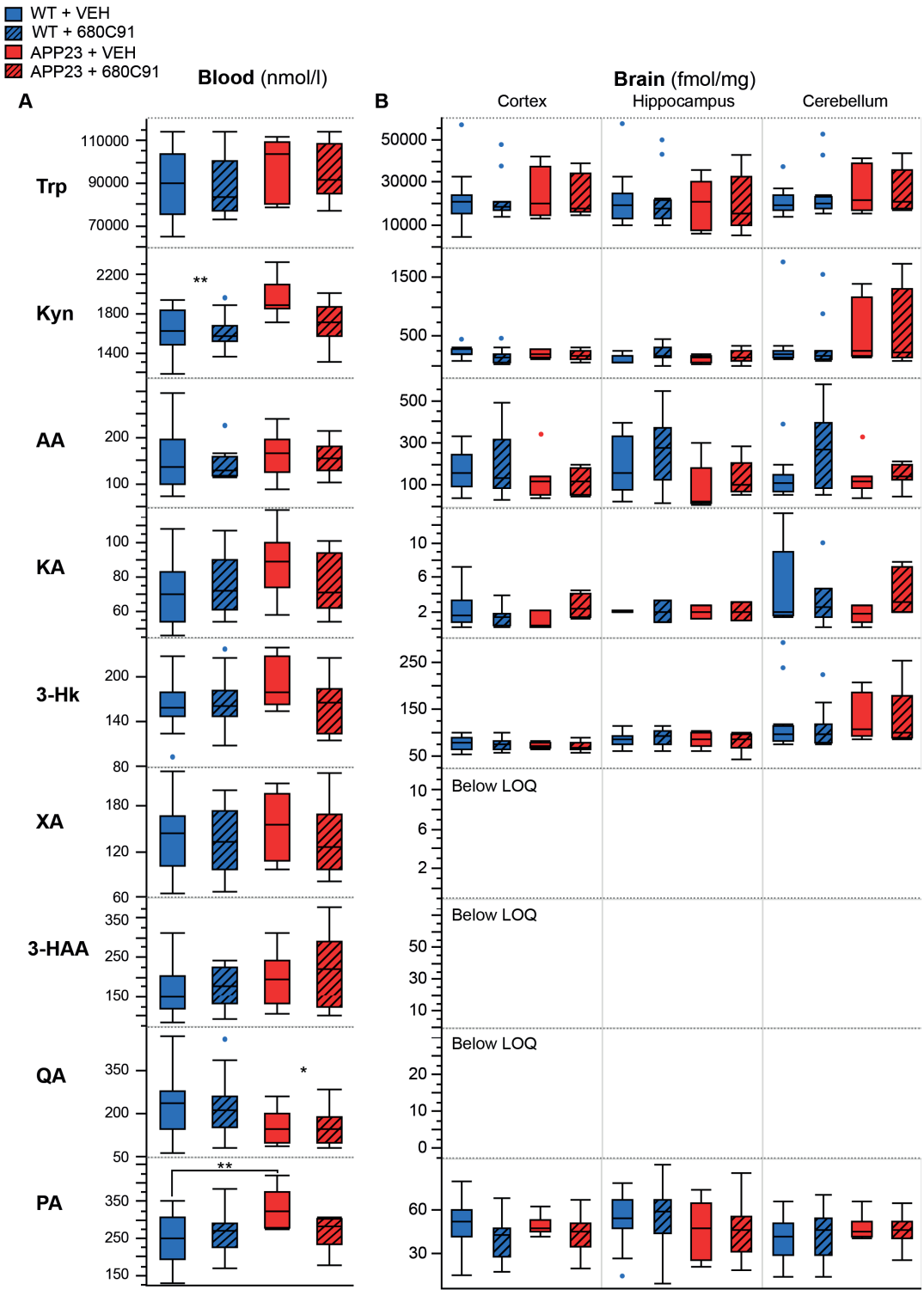
Results from the open field test, which was performed to analyse general exploratory behaviour, indicated that VEH- and 680C91-treated APP23 and wild-type mice spent a similar amount of time in the centre circle and corners of the open field set-up (**Figure 2E**).

Next, to more specifically address anxiety-related behaviour mice were tested in the elevated plus maze (**Figure 2F**). Following up on a significant treatment-by-genotype interaction effect ($F(3,40)=5.9$, $p=.020$), post-hoc analyses indicated a trend towards reduced closed arm visits after 680C91 treatment in APP23 mice ($p=.060$), which was not found in wild-type mice ($p=.739$). Further analyses indicated that, irrespective of treatment, APP23 mice spent less time in the closed arms ($F(1,40)=9.7$, $p=.004$) and more time in the open arms of the maze ($F(1,40)=10.3$, $p=.003$), while also visiting the open arms more frequently than wild-type mice ($F(1,40)=6.8$, $p=.013$)

Taken together, these data suggest that APP23 mice show reduced anxiety-related behaviour which is not affected by long-term oral treatment with a TDO inhibitor.

**► Figure 3. Inhibition of tryptophan 2,3-dioxygenase has minor effects on kynurenine metabolites in blood and does not affect kynurenine metabolites in the brains of APP23 mice**

**A.** Boxplots showing distributions of Kyn pathway metabolites in blood and (**B**) cortex, hippocampus and cerebellum from 6-month-old wildtype and APP23 mice after a 6-week treatment with vehicle (DMSO) or vehicle + 680C91 through oral gavage (n=8-13). Significance of the main effect of genotype in two-way ANOVA or Tukey post-hoc tests (in case of significant interaction) are depicted with * $p<.05$ or ** $p<.01$. **Abbreviations**: Trp, Tryptophan; Kyn, Kynurenine; AA, anthranilic acid; KA, kynurenic acid; 3-Hk, 3-hydroxykynurenine; XA, xanthurenic acid; 3-HAA, 3-hydroxyanthranilic acid; QA, quinolinic acid; PA, picolinic acid.


WT + VEH
WT + 680C91
APP23 + VEH
APP23 + 680C91
A
Blood (nmol/l)
B
Brain (fmol/mg)
Cortex
Hippocampus
Cerebellum
Trp
Kyn
AA
KA
3-Hk
XA
3-HAA
QA
PA
Below LOQ
Below LOQ
Below LOQ
**
**
*

### Long-term TDO inhibition has a minor influence on kynurenines in serum of APP23 mice and does not affect brain kynurenines

Finally, to investigate whether TDO inhibition could have improved recognition memory in APP23 mice by modulating the Kyn pathway, we analysed Kyn metabolites in serum and brain tissue of the VEH- and 680C91-treated APP23 and wild-type mice that had undergone behavioural tests.

The analyses indicated increased serum concentrations of Kyn in APP23 mice (genotype-effect: $F(1,40)=10.3$, $p=.003$) (**Figure 3A**) while a trend was observed towards reduced Kyn in serum after treatment with 680C91 (treatment-effect: $F(1,40)=4.0$, $p=.052$). Concentrations of QA were reduced in APP23 mice ($F(1,40)=5.2$, $p=.029$) and, following up on a significant genotype-by-treatment interaction effect ($F(1,40)=4.67$, $p=.037$), post-hoc analyses indicated increased PA concentrations in VEH-treated APP23 mice compared to VEH-treated wild-type mice ($p=.024$) whereas concentrations did not differ between 680C91-treated APP23 or wild-type mice and VEH-treated wild-type mice ($p=.914$ and $p=.842$). These data suggest that long-term oral treatment with 680C91 specifically lowers PA concentrations while possibly reducing the increased Kyn concentrations in serum of APP23 mice.

680C91 treatment did not influence the concentrations of Kyn metabolites in the cortex, hippocampus or cerebellum (**Figure 3B**). In addition, there were no differences between APP23 and wild-type mice and no interaction effects.

Taken together, these results show that long-term treatment with the TDO inhibitor 680C91 has minor effects on Kyn metabolite profiles in blood of APP23 and wild-type mice and does not affect Kyn metabolite levels in the cortex, hippocampus and cerebellum.

## Discussion

The Kyn pathway has been frequently linked to AD because of its involvement in aging, inflammation and neurotoxicity (Maddison and Giorgini 2015). Several studies have indicated that kynurenines could provide biomarkers for AD (Giil et al. 2017; Gulaj et al. 2010; Hartai et al. 2007; Widner et al. 2000a) and that Kyn pathway enzymes, including TDO, could potentially be targeted to prevent or delay amyloid-beta-induced cellular toxicity (van der Goot et al. 2012; Breda et al. 2016). To further explore the role of the

Kyn pathway in aging and AD, we made use of the APP23 amyloidosis mouse model to investigate the effect of aging on Kyn profiles and the effect of long-term TDO inhibition on cognitive function. We found reduced serum concentrations of several downstream Kyn metabolites in APP23 mice. Independent of genotype, aging strongly affected metabolite levels in serum and was associated with region-dependent variations in brain tissue, most prominent in the hippocampus. Oral administration of the TDO inhibitor 680C91 rescued impairments in recognition memory in APP23 mice without altering spatial learning and memory or anxiety-related behaviour.

In accordance with studies in AD patients (Hartai et al. 2007; Giil et al. 2017; Gulaj et al. 2010; Heyes et al. 1992), we found that multiple Kyn metabolites downstream of Kyn were reduced in serum of APP23 mice. The mechanisms underlying these changes are unknown but could include increased metabolism or disturbances in the circadian rhythm - which have both been previously observed in APP23 mice (Vloeberghs et al. 2004; Vloeberghs et al. 2008). Such disturbances could affect Trp metabolism that shows a circadian pattern (Rapoport and Beisel 1968; Coggan et al. 2009; Carpenter et al. 1998; Kennedy et al. 2002),. As alterations in the circadian rhythm are common in AD patients and are closely related to metabolic dysfunction in AD pathology (Mattis and Sehgal 2016), we speculate that reduced downstream Kyn pathway activity in AD patients or APP23 mice might reflect a state of metabolic and/or circadian dysregulation.

To our knowledge, this is the first report on the effect of aging on kynurenines in the blood of mice. In line with studies in humans (Heyes et al. 1992; Giil et al. 2017), we found that QA accumulates during aging in blood of mice. Aging was recently shown to be associated with impaired metabolism of QA towards NAD+ in human macrophages, which significantly impacted their immune function (Minhas et al. 2018). Similarly, hampered QA-dependent de novo synthesis of NAD+ led to dysregulated cellular homeostasis in mouse models of hepatic and renal damage (Poyan Mehr et al. 2018; Katsyuba et al. 2018). We speculate that QA accumulation in the blood could represent a cross-species phenotype of aging indicative of maladaptive cellular responses to age-related damage. Differences between mice and men with regard to age-related changes include a lack of increase of Kyn in serum of mice (Rist et al. 2017; Theofylaktopoulou et al. 2013; Collino et al. 2013; Yu et al. 2012; Capuron et al. 2011; Niinisalo et al. 2008; Pertovaara et al. 2006;

Frick et al. 2004). This could be attributed to species-specific activity of Kyn pathway enzymes (Fujigaki et al. 1998).

Our data indicated no alterations of Kyn metabolites in brain tissue of APP23 mice. This is in contrast with previous work on the Kyn pathway in AD mice (Zwilling et al. 2011; Wu et al. 2013) and could result from the use of different AD mouse models and the extend of brain pathology. In line with evidence in AD patients (Heyes et al. 1992), one study showed reduced KA concentrations in the cortex of 7-month-old J20 mice (Zwilling et al. 2011). In contrast to APP23, expression of mutated APP is driven by a promotor that is expressed during embryonic development (Mucke et al. 2000).This could alter embryonic Kyn pathway activity and modulate KA concentrations in the brain in later life (Pershing et al. 2015). Another study analysed Kyn pathway activity in the brains of 3-, 6- and 12-month old triple transgenic mice (3xTg□AD) (Wu et al. 2013) that overexpresses mutant APP and MAPT (encoding tau protein) on a presenilin-1 (PSEN1) knock-in background (Oddo et al. 2003). They demonstrated increased QA levels in the hippocampus of transgenic mice that progressed with aging. As we were unable to detect QA in our brain samples, a similar increase of QA levels in our model seems unlikely. As QA is mainly produced by microglia in the brain (Heyes et al. 1996; Guillemin et al. 2003), these results could be interpreted in line with evidence of age-dependent microglial activation in the 3xTg□AD model (Belfiore et al. 2019). However, as QA concentrations are not increased in post-mortem brain tissue of AD patients (Moroni et al. 1986; Mourdian et al. 1989; Sofic et al. 1989), Kyn pathway disturbances in 3xTg□AD might not be related to AD pathology. The above considerations should be taken into account when studying the Kyn pathway in AD mouse models.

In line with studies showing that TDO inhibition can improve AD-related behavioural deficits (Woodling et al. 2016; Too et al. 2016), we demonstrated that long-term oral administration of the TDO inhibitor 680C91 restored recognition memory deficits in APP23 mice. Treatment with 680C91 was previously shown to inhibit cerebral TDO activity (Cuartero et al. 2014). However, and although 680C91 affected Kyn levels in the serum, we did not observe a treatment effect on Kyn pathway activity in the brains of AD mice. We therefore speculate that TDO might have a role in brain physiology that is independent of its enzymatic function. Indeed, TDO inhibition reduced Aβ toxicity independent of Kyn metabolites in C. elegans (van der Goot et al. 2012). Alternatively, as

TDO expression is highly restricted to subregions in the brain, TDO inhibition could impact cellular function without causing measurable changes in metabolite concentrations at a regional level. Of interest in this regard, is the fact that TDO seems to be integrated in damage response mechanisms in the brain that are regulated by stress signals such as glucocorticoids and prostaglandins (Brooks et al. 2016; Ochs et al. 2015; Dostal et al. 2017) which might be activated by Aβ (Woodling et al. 2016). To establish whether TDO inhibition indeed offers therapeutic potential in AD, mechanistic studies should address how TDO activation impacts neuronal functioning and how this might influence AD-related cognitive dysfunction.

We provided an extensive analysis of kynurenine profiles in serum and brain tissue during aging in a mouse model of AD and a characterization of the effects of pharmacological TDO inhibition on the Kyn pathway and cognitive function. However, our results should be discussed in light of the studies limitations. First of all, we observed differences in Kyn profiles between untreated and treated groups of mice (see **Figure 1** and **Figure 2**). These differences are possibly explained by differences in the time of the day at which mice were. Secondly, contrary to previous reports from our group (Van Dam et al. 2003), spatial learning and memory were not impaired in 6-month-old APP23 mice as assessed in the Morris water maze. However, in the current study, mice were handled on a daily basis by a single experimenter during three weeks prior to the start of behavioural tests. Although chronic stress protocols can hamper mouse performance in the Morris water maze (Moreira et al. 2016), daily handling of mice and habituation to the experimenter can reduce stress-related behaviour and improve cognitive function (Neely et al. 2018; Hurst and West 2010). The development of novel TDO inhibitors that show higher solubility and stability will allow for the use of treatment routes such as food pellets or osmotic pumps that reduce such experimental biases.

In conclusion, this study revealed age-related and genotype-specific changes of the Kyn pathway in the APP23 mouse model of AD. As these changes were partially in accordance with studies in humans, the APP23 mouse model might be used to study the role of the Kyn pathway in AD. Furthermore, we demonstrated that long-term inhibition of TDO improved hippocampal-based recognition memory in APP23 mice without majorly influencing central measures of Kyn pathway activity. These data suggest that

Kyn pathway activation could be a cross-species aging phenotype and warrants further investigation of the role of TDO in AD pathophysiology.

## Acknowledgements

The authors would like to thank Jana Janssens, Jan van Erum, Elke Calus and Tinne Koninckx for assisting in the breeding and sacrificing of the mice used in this study. This work was supported by the Research Foundation-Flanders (FWO), Interuniversity Poles of Attraction of the Belgian Federal Science Policy Office (IAP7/16), Special Research Fund UAntwerpen (Bijzonder Onderzoeksfonds, BOF, # 34423), agreement between Institute Born-Bunge and University of Antwerp, the Alzheimer Research Centre Groningen (UMCG, The Netherlands), the Medical Research Foundation Antwerp, the Thomas Riellaerts research fund, and Neurosearch Antwerp.

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